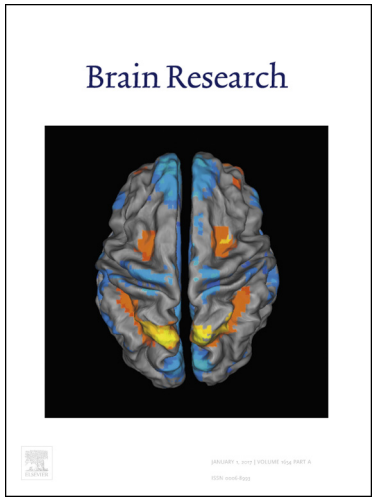

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Invited Review

Visceral pain – Novel approaches for optogenetic control of spinal afferents

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Abstract

Painful stimuli arising within visceral organs are detected by peripheral nerve endings of spinal afferents, whose cell bodies are located in dorsal root ganglia (DRG). Recent technical advances have made it possible to reliably expose and inject single DRG with neuronal tracers or viruses in vivo. This has facilitated, for the first time, unequivocal identification of different types of spinal afferent endings in visceral organs. These technical advances paved the way for a very exciting series of in vivo experiments where individual DRG are injected to facilitate opsin expression (e.g. Archaerodopsin). Organ-specific expression of opsins in sensory neurons may be achieved by retrograde viral transduction. This means activity of target-specific populations of sensory neurons, within single DRG, can be modulated by optogenetic photo-stimulation. Using this approach we implanted micro light-emitting diodes (micro-LEDs) adjacent to DRG of interest, thereby allowing focal DRG-specific control of visceral and/or somatic afferents in conscious mice. This is vastly different from broad photo-illumination of peripheral nerve endings, which are dispersed over much larger surface areas across an entire visceral organ; and embedded deep within multiple anatomical layers. Focal DRG photo-stimulation also avoids the potential that wide-field illumination of the periphery could inadvertently activate other closely apposed organs, or co-activate different classes of axons in the same organ (e.g. enteric and spinal afferent endings in the gut). It is now possible to selectively control nociceptive and/or non-nociceptive pathways to specific visceral organs in vivo, using wireless optogenetics and micro-LEDs implanted adjacent to DRG, for targeted photo-stimulation.

Key Words:

Adeno-associated Virus (AAV), nociception, Visceral pain, spinal afferent, optogenetics, channelrhodopsin, Archaerodopsin, DRG, pain, wireless optogenetics. Light emitting diodes (LEDs), optogenetics, sensory neuron, Trpv1.

Introduction

Pain is an evolutionary adaptation to avoid potential or actual tissue damaging events (Smith and Lewin, 2009). Painful sensations are initiated by specific classes of sensory neurons that can discriminate noxious stimuli, from physiologically-innocuous stimuli. Sensory neurons that can respond to noxious stimuli are called nociceptors (Perl, 2007). Compared to somatic pain, visceral pain is typically a diffuse and poorly localized sensation (Cervero and Laird, 1999) and the mechanisms underlying transduction of visceral pain are poorly understood (Gebhart and Bielefeldt, 2016). Indeed, the different morphological types of somatic nociceptor endings have been well characterized, particularly in skin (Mense, 2009). In contrast, the distribution and morphologies of the sensory nerve endings that underlie detection of visceral pain remains mysterious. Recently, major technical developments have allowed unequivocal identification of the spinal afferent nerve endings that underlie murine visceral sensation to be characterized (Kyloh and Spencer, 2014, Spencer et al., 2016c). This technique has also allowed a major new series of experiments to be undertaken using wireless optogenetics to control visceral sensations. These advances will be discussed below.

Sensory pathways innervating visceral organs

In vertebrates, two major sensory pathways innervate the body, below the head. These are the vagal and spinal afferents, which provide the vast majority of sensory innervation to visceral and somatic organs (Gebhart and Bielefeldt, 2016). These two sensory pathways have distinct origins and encode different stimuli (Powley et al., 2005, Powley et al., 2015, Brierley et al., 2004). The nerve cell bodies of vagal afferents arise from nodose and jugular ganglia, while spinal afferents arise from DRG (Gebhart and Bielefeldt, 2016). While some evidence suggests that the vagus nerve can respond to nociceptive stimuli (Yu et al., 2005), there is a long held belief that vagal afferents play little, or no role, in the transmission of visceral pain sensations that arise from internal organs (Gebhart and Bielefeldt, 2016). Indeed, there is no doubt that the vast majority, if not all visceral pain is transmitted via spinal afferents, whose sensory pathways enter the dorsal horn of the spinal cord (Gebhart and Bielefeldt, 2016). This is supported by selective lesions to particular spinal afferent pathways which abolishes noxious responses from visceral organs, like the large intestine (Kyloh et al., 2011). For this

reason, spinal afferents are of supreme interest to understand the mechanisms of visceral nociception (Gebhart and Bielefeldt, 2016).

Overcoming technical obstacles to identify spinal afferent endings

The major obstacles to identification of visceral spinal afferent nerve endings has been their positive discrimination from other sensory and motor nerve axons, which travel along the same mixed nerve trunks. Disentangling afferent from efferent axons and their nerve endings within visceral organs has been exceptionally challenging. In the gastrointestinal tract, identifying spinal afferents has been even more challenging than in other visceral organs, because the gut's own independent nervous system (Furness, 2006b, Furness, 2006a), the enteric nervous system, contains many neurochemical markers also expressed in DRG neurons. These include neuropeptides such as Calcitonin Gene Related Peptide (CGRP) and substance P (Costa et al., 1996).

Unlike spinal afferents, whose endings have only recently been identified with selective tracing methods, the terminals of vagal afferent neurons have been well described in viscera (Powley, 2000, Powley et al., 2013, Powley and Phillips, 2011). This is largely due to the comparative ease of accessing the nodose ganglia using surgical approaches (Powley, 2000, Powley et al., 2013, Powley and Phillips, 2011). Injection of neuronal tracers into the nodose ganglia enables selective unambiguous anterograde labelling of vagal afferent nerve terminals (Powley, 2000, Powley et al., 2013).

Development of anterograde tracing to selectively label spinal afferents in visceral organs and their nerve endings

The recent technical development of selective anterograde tracing from DRG has enabled selective labeling of spinal afferent axons and endings (Spencer et al., 2014, Spencer et al., 2016a). Using this technique, numerous spinal afferent endings, most of which had never been previously unidentified, were labelled in the large intestine (Spencer et al., 2014), stomach and esophagus (Spencer et al., 2016a), and urinary bladder (Spencer et al., 2018). Some, or all of the different types of endings identified must contribute to the sensation of pain, because lesions to spinal afferent pathways abolishes pain transmission from organs like the gut (Kyloh et al., 2011). Exposing DRG as a survival surgery in mice is now routine in our laboratory and has revealed significant new information on the morphology, neurochemical coding and sites of innervation of spinal afferents (Spencer et al., 2016c). One of the major benefits of this technique is that reducing quantities of injected tracer to very small amounts often results in labelling of single spinal afferent axons and their associated endings. This enables visualization of single afferent nerves, without contamination by other axons in the field of view

(Spencer et al., 2014, Spencer et al., 2016a). This enabled the first unequivocal correlation of the outputs (endings) arising from a single spinal afferent axon in viscera (Fig.1).

Use of transgenic reporter mice – can they identify spinal afferent nerve endings?

Recently, we generated a transgenic mouse in which the fluorescent reporter mCherry was knocked into the CGRPα locus (Hibberd et al., 2016). This had also been performed using different reporter genes by earlier investigators (McCoy et al., 2012). CGRPα is highly expressed in DRG neurons, making it particularly a useful marker (Mulderry et al., 1988). This mouse has proved very useful for the visualization of peptidergic spinal afferent neurons in live DRG and visceral organs (Hibberd et al., 2016). However, the morphological resolution of CGRPα expressing axons was low in visceral organs and precise morphological features of spinal afferents could not be determined (Spencer et al., 2016b). Nevertheless, the reporter (mCherry) signal enabled recordings from individual varicosities of spinal afferent axon terminals within live visceral organs, such as the gut wall) (Spencer et al., 2016b), and was also useful in reporting the functional properties of the peptidergic and non-peptidergic sensory nerve cell bodies in live tissue (Hibberd et al., 2016).

Optogenetic control of spinal afferent pathways

Recent studies have demonstrated the effectiveness of optogenetics in the selective activation of specific classes of visceral afferents *in vitro* (Feng et al., 2016). These studies showed that different functional classes of spinal afferents from the mouse large intestine can be identified by applying focal pulses of blue light to isolated segments of distal colon *in vitro* (Feng et al., 2016).

A challenge for optogenetic illumination of visceral organs *in vivo* has been how to selectively control light penetration to deep visceral organs without spread to neighboring organs. Our experience has led us to believe that there are significant advantages for optogenetic control of visceral organs if light is delivered to visceral sensory neurons at the DRG rather than at the peripheral nerve endings within the organ. We have been working to develop wirelessly-activated micro-LEDs with appropriate dimensions to deliver blue or green pulses of light directly onto DRG sensory nerve cell bodies to control visceral sensation in conscious mammals. To do this, the micro-LEDs were developed with specific dimensions to implant adjacent to DRG during survival surgery (Fig.2). There are multiple advantages of implanting micro-LEDs adjacent to DRG. Firstly, the sensory nerve cell bodies are concentrated into a ~1mm diameter region of tissue (in mice), where deep penetration of light is not required. This contrasts with their peripheral nerve endings which are located long distances away from nerve cell bodies innervate much larger spatial fields. Secondly, DRG consist only of spinal

afferent neurons and lack synaptic inputs. Therefore, since no other classes of sensory or motor nerve axons arise from DRG, or provide *en passant* innervation to DRG, this means direct illumination of DRG with micro-LEDs provides selective control of spinal afferent neurons. This contrasts with light delivery in peripheral visceral organs, where multiple functional classes of axons can express the same genes and may cause inadvertent co-activation. The gut wall for example, contains both spinal afferent nerve endings (Mulderry et al., 1988, Hibberd et al., 2016) and enteric neurons that express CGRP (Furness et al., 2004). Therefore, photostimulation of the gut wall could indiscriminately activate spinal afferents and enteric nerves (Mulderry et al., 1988, Hibberd et al., 2016). Also, delivering light to any periphery organ would require illumination of a larger spatial field compared with individual DRGs, which in mice are approximately 1mm in diameter. Finally, when illuminating light to any peripheral organ, it can be problematic preventing dispersed light from inadvertently activating other neighboring organs (if they also express the opsins). For these reasons, we felt direct illumination of DRG sensory nerve cell bodies with micro-LEDs represents a number of conspicuous advantages in terms of targeting pure visceral sensory pathways.

We have recently implanted single or multiple micro-LEDs (green or blue) adjacent to DRGs of interest, with no cases of infection or rejection by the animal. We have demonstrated in pilot experiments that at least 4 miniature micro-LEDs can be activated simultaneously, via wireless technology (Fig.2). Prior to implantation of the micro-LEDs, single DRGs are injected with cre-inducible adeno-associated viruses that lead to opsin expression. In transgenic TRPV1-cre mice, it was found that after 4-5 weeks post DRG viral injection of a cre-inducible AAV (AAV5-EF1a-DIO-hCHR2(H134R)-eYFP) into single DRG, robust expression of yellow fluorescent protein (YFP) developed in numerous TRPV1-immunoreactive, cre-expressing sensory neurons (Fig.3). This approach of injecting AAVs directly into DRGs is an alternative strategy for generating expression of opsins in DRG neurons exclusively.

Evidence for optogenetic control of visceral sensation

Although wireless optogenetics has been convincingly demonstrated to control somatic sensation and behaviour (Montgomery et al., 2015), only recently, has this technology been used to effectively control visceral sensation (Samineni et al., 2017). This recent work has shown that in transgenic mice expressing the light-activated inhibitory proton pump, Archaerhodopsin (under the control of the sensory-neuron specific sodium channel (sns) promoter), photo-stimulation effectively silenced bladder afferents underlying the visceromotor response *in vivo* (Samineni et al., 2017). This is sound evidence that visceral pain pathways can be effectively silenced using wireless photo-stimulation of Archaerhodopsin.

Optogenetic control of spinal afferent pathways to specific organs in conscious non-transgenic animals

Of particular interest to our laboratory is selective, visceral organ-specific control of nociceptive and non-nociceptive pathways in non-transgenic animals. This has been successfully demonstrated for somatic nociception in non-transgenic mice (Iyer et al., 2014) and rats (Boada et al., 2014). In the study by Iyer et al. (2014), the adeno-associated virus AAV6-hSyn-eNpHR3.0-eYFP was injected into the sciatic nerve, which was found to retrogradely transport successfully to DRG, leading to sufficient expression of halorhodopsin in sensory neurons. These investigators then showed that transdermal yellow light applied to the hind-limb reduced acute pain perception, reversed mechanical allodynia and reversed thermal hyperalgesia in a neuropathic pain model (Iyer et al., 2014). These experiments were possible because light was delivered transdermally (to an external organ), so that pain responses could be induced in freely moving animals. However, delivering light focally, to specific internal organs, in conscious, freely-moving, untethered animals is more difficult. To get around this, micro-LEDs can be implanted and fixed adjacent to DRG in non-transgenic animals (Fig.3A) after injection of AAVs in visceral organ(s) of interest. In this scenario, target-specific visceral afferents can be controlled directly at the DRG level, without illuminating the organ itself. In another study, transgenic mice expressing Archerodopsin under the control of the Nav1.8 promoter, it was found that acute transdermal illumination of the hind paws led to a clear reduction in allodynia to mechanical stimuli under inflammatory conditions (Daou et al., 2016). The study also showed that basal mechanical sensitivity was not affected by the illumination (Daou et al., 2016).

Another possibility for wireless optogenetic control of DRG in non-transgenic animals is to use two different viruses to express opsins in DRG. This can be performed by first injecting the canine adenovirus-2 (Cav-2) into the target organ of interest (e.g. gut) (Fig.2) which transports readily retrograde along axons (Junyent and Kremer, 2015). Then, a cre-inducible AAV is injected into the DRG of interest, such that only the sensory neurons that project to the target organ will express the opsins. Implantation of micro-LEDs at the DRG could then be used to selectively control visceral afferent pathways to specific organs in vivo, in non-transgenic animals.

Advantages of wireless optogenetic photo-illumination of DRG to selectively control visceral afferent pathways in conscious animals

- Specific populations of sensory neurons projecting to particular visceral organs (e.g. GI-tract or bladder) can be selectively activated, or silenced at particular DRG levels of interest.

- Selective activation or silencing of individual DRGs can be used to determine the functional contribution of each vertebral segment to nociceptive responses.
- Direct photo-stimulation of DRG can only activate spinal afferents - since no other classes of sensory or motor nerve cell bodies (or axons) reside in, or pass through DRG. This avoids the potential concern arising with illumination of the mixed neural innervation of peripheral organs, where concomitant activation of other functional classes of sensory or motor axons can occur.
- Direct DRG photo-stimulation is highly focused (~1mm^2) and avoids the need for broad illumination of large surface areas in peripheral target organs. This also reduces potential for inadvertent non-specific illumination of neighboring organs.
- Multiple micro-LEDs can be wirelessly activated at the same time at different DRG levels (Fig.2).
- DRG photo-stimulation enables direct modulation of spinal afferent cell bodies, requiring a shallow depth of photo-penetration (~100-200µm). Achieving full activation of all populations of spinal nerve endings throughout the full depth of peripheral organs in viscera requires considerably deeper photo-penetration. [Spinal afferent endings have been shown to have varying depths of innervation in viscera, commonly 1-2mm deep in gut or bladder].
- Spinal afferent pathways to specific *visceral* organs could be readily activated or silenced in *non-transgenic* animals, using the described technique of direct DRG illumination.

Disadvantages of wireless optogenetic photo-illumination of DRG to selectively control visceral afferent pathways in conscious animals

- It requires surgery to implant the micro-LEDs.
- There is always possibility of rejection or infection with implanting foreign bodies *in vivo*.
- Multiple micro-LEDs would be required to be implanted at multiple vertebral segments to completely silence visceral pain pathways, which are known to enter the spinal cord at multiple vertebral segments.

Differences between chemogenetics and optogenetics for control of visceral nociception

There is gathering interest in the use of designer receptors exclusively activated by designer drugs (DREADDs) to control sensory pathways (including those of nociceptive origin) in genetically defined cell populations, both *in vitro* and *in vivo*. Both excitatory G-protein (Gq)-

DREADD (hM3Dq) and the inhibitory G-protein (Gi)-DREADD (hM4Di) exist which are under exclusive control of the biologically inert compound clozapine N-oxide (CNO). There are some significances in the mechanisms of DREADD technology to study visceral pain compared with optogenetics. For example, DREADDs are advantageous for studying phenotypes of neurons that are diffusely scattered in multiple organs. Therefore, CNO application penetrates a wider area than can be achieved with optogenetics, where neurons only in the immediate arc of light are activated. Unlike optogenetics, which allows fast and reversible control of neuron activity, manipulation of hM3Dq and hM4Di receptors affects neuronal activity over many minutes to hours. A recent study has demonstrated that Gi-DREADD expression in peripheral nerves can produce a ligand-dependent analgesia to heat *in vivo* and also reduced neuronal firing in single-cells *in vitro* (Saloman et al., 2016). Interestingly, however, the authors revealed that expression of Gi-DREADD also caused ligand-independent changes in ion channel activity and second-messenger signaling, highlighting both the potential and the limitations of DREADD technology.

Conclusions

The recent development that single DRG in mice can now be reliably exposed and injected with viruses, or neuronal tracers as a survival surgery, is a major step forward in our ability to visualize and selectively control spinal afferent pathways to specific visceral organs. This advance means that we can not only identify the location and morphology of spinal afferent endings, but we can selectively express opsins in particular classes of neurons in specific DRGs. The advantage of exposing single DRGs has the major benefit of being able to implant micro-LEDs adjacent neuronal cell bodies facilitating direct wireless activation or inhibition of visceral or somatic afferent pathways. The micro-LEDs we developed have specific dimensions compatible with implantation adjacent to single DRG in mice and can be modified readily for rats.

There is no doubt there is a desperate need for alternative techniques to suppress chronic pain. In this regard, there is significant interest in the pursuit of alternative techniques to suppress nociception, without drugs. Wireless optogenetic illumination of specific populations of sensory nerve cell bodies in DRG is an exciting reality for selective control of visceral pain pathways to organs of interest.

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FIGURE LEGENDS

Figure 1.

Novel technique to selectively label spinal afferent axons and nerve endings in visceral organs. During anesthesia, a midline incision is made in the dorsal surface to expose relevant DRG. A glass micropipette filled with dextran biotin is injected into single DRG of interest. Because DRG contain only the sensory neurons of spinal afferents, this means that only spinal afferent axons and endings will be selectively labeled in the target visceral organs. A major advantage of this technique is that it simultaneously labels spinal afferents in the urinary bladder, colon and uterus, when injections are made into the lumbosacral DRGs. B, shows a single spinal afferent axon and diverse morphological types of endings in the detrusor muscle of the mouse urinary bladder. C, shows a complex-type spinal afferent ending in the urinary bladder. D, shows a single spinal afferent axon in the detrusor muscle branching into multiple complex-type endings.

Figure 2.

Development of wireless optogenetics for control of visceral pain. A, resonant cavity with micro blue LED activated. B, green LED for activation of Archaerhodopsin in DRG sensory neurons. C, micro LED and receiver, built in house. The relative size of the micro LED is shown adjacent to a match head. D, demonstration that multiple blue and green micro LEDs can be activated simultaneously via wireless resonant cavity. E, circuit board of wireless micro LED. F, Diagrammatic representation of the technique to suppress nociception selectively from target visceral organs. Firstly, cre-inducible AAV in a double-floxed inverted orientation expressing Channelrhodopsin and yellow fluorescent protein (e-YFP) is injected into single DRG at relevant vertebral level. Then a cre-inducible AAV is injected into the target organ of interest. This facilitates retrograde transport of cre back into only the DRG sensory neurons that project to the colon. Therefore, this means that Archaerhodopsin or Channelrhopsin will be expressed in only colon-projecting sensory neurons. Therefore, when green or blue LEDs are illuminated onto DRGs at the relevant location, this means that colon-projecting sensory neurons can be selectively activated or inhibited.

Figure 3.

Technique to implant wireless micro-LEDs adjacent to DRGs for silencing visceral pain. A, cartoon representing the site of implanting micro LEDs adjacent to DRGs in the lumbosacral region of spinal cord to control visceral nociception to the lower gastrointestinal tract. Blue or green LEDs surgically implanted adjacent to DRGs can be used to wirelessly activate or inhibit colon-projecting sensory neurons of selectively. B, in a TRPV1-cre mouse line (Lagerstrom et al., 2010), injection of AAV5-DIO-Arch 3.0-eYFP generates yellow fluorescent protein expression in TRPV1+ve sensory neurons. Image shows a whole mounted TRPV1-Cre positive DRG (left L6 and S1) injected with rAAV5/EF1a-DIO-eArch3.0-eYFP to induce

expression of Archaerhodopsin. B, shows TRPV1 immunoreactivity from L6 and S1 DRG, C, show the same image in B with YFP expression 5 weeks after injection of 50nL of active rAAV5/EF1a-DIO-eArch3.0-eYFP into L6 and S1 DRG. D, show co-localization of both YFP and TRPV1 expression from B and C, respectively. Red arrows indicate areas of co-localisation. Scale bar, 100µm.

Highlights

- Recent studies have now demonstrated that anterograde tracing can be reliably performed from dorsal root ganglia (DRG) *in vivo,* to identify single spinal axons and their associated nerve endings, in visceral organs.
- Channelrhodopsin or Archaerhodopsin can now be selectively expressed in single DRG, following viral injections into single DRG *in vivo*.
- Wireless micro-light emitting diodes (micro-LEDs) can now be implanted adjacent to single DRG in live mice.
- Visceral sensation, including nociceptive and non-nociceptive pathways, can now be selectively controlled to specific target organs, by wireless optogenetics, via photo-stimulation at the level of single DRGs.
- There are a number of significant advantages of photo-stimulation at the DRG level *in vivo* for wireless optogenetic control of visceral and/or somatic sensation, compared with broad photo-stimulation of large surface areas of peripheral organs.